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Systemic and local effects of long-term exposure to alkaline drinking water in rats

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Summary. Alkaline conditions in the oral cavity may be caused by a variety of stimuli, including tobacco products, antacids, alkaline drinking water or bicarbonate toothpaste. The effects of alkaline pH on oral mucosa have not been systematically studied. To assess the systemic (organ) and local (oral mucosal) effects of alkalinity, drinking water supplemented with $Ca(OH)_2$ or NaOH, with pH 11.2 or 12 was administered to rats ($n = 36$) for 52 weeks. Tissues were subjected to histopathological examination; oral mucosal biopsy samples were also subjected to immunohistochemical (IHC) analyses for pankeratin, CK19, CK5, CK4, PCNA, ICAM-1, CD44, CD68, S-100, HSP 60, HSP70, and HSP90.

At completion of the study, animals in the study groups had lower body weights (up to 29% less) than controls despite equal food and water intake, suggesting a systemic response to the alkaline treatment. The lowest body weight was found in rats exposed to water with the highest pH value and starting the experiment when young (6 weeks). No histological changes attributable to alkaline exposure occurred in the oral mucosa or other tissues studied. Alkaline exposure did not affect cell proliferation in the oral epithelium, as shown by the equal expression of PCNA in groups. The up-regulation of HSP70 protein expression in the oral mucosa of rats exposed to alkaline water, especially $Ca(OH)_2$ treated rats, may indicate a protective response. Intercellular adhesion molecule-1 (ICAM-1) positivity was lost in 6/12 rats treated with $Ca(OH)_2$ with pH 11.2, and loss of CD44 expression was seen in 3/6 rats in both study groups exposed to alkaline water with pH 12. The results suggest that the oral mucosa in rats is resistant to the effects of highly alkaline drinking water. However, high alkalinity may have some unknown systemic effects leading to growth retardation, the cause of which remains to be determined.

Keywords: drinking-water, alkaline, oral mucosa, rat

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Table 1. Study groups

Group	Treatment	pH of drinking water	Number of animals	
			Female	Male
1	None (control)	pH 7	6	6
2	Ca(OH)2	pH 11.2	6	6
3	NaOH	pH 11.2	6	6
4	Ca(OH)2	pH 12	3	3
5	NaOH	pH 12	3	3

Introduction

Oral mucosal health is affected by nutritional state and exposure to various extrinsic factors such as micro-organisms and chemicals. As a mucous barrier, saliva has an important protective function against a variety of potential noxious agents, toxins, and hazardous chemicals (Tabak *et al*. 1982; Mandel 1987). The pH of the oral cavity is maintained as relatively neutral by the buffer capacity of saliva (Mandel 1987). Acidic changes in the oral cavity due to bacterial metabolism, acidic drinks, and reflux are well known. Changes towards alkaline conditions, on the other hand, have received much less attention. However, an alkaline shift in the extracellular environment may influence the growth properties of cells (Eagle 1973), and short-term alkaline stress may even cause proliferative activity (Zetterberg & Engström 1981).

Transient alkalinity of the oral cavity may be brought about by a variety of factors, e.g. tobacco products, currently considered the most important causative factors of oral cancer. Cigar smoke is generally more alkaline than cigarette smoke (Brunnemann & Hoffmann 1972; von Kirsch 1963). The pharmacological effects of tobacco are related to alkalinity, due to the enhanced absorption of nicotine through the mucosa in a free-base form (Tomar & Henningfield 1997). Smokeless tobacco products (e.g. snuff), popular in Scandinavia and the US, have high alkaline pH (Axell 1993), regulated by buffering agents (Hirsch 1983; Tomar & Henningfield 1997; Gothia Tobak 1999). The Sudanese smokeless tobacco product *toombak* (with a pH of about 9.6) may cause oral mucosal changes at least partly attributable to its alkalinity (Idris *et al*. 1996). Betel-nut chewing associated with the use of lime paste, mainly consisting of calcium hydroxide and seasoning additives (Jeng *et al*. 1994), rapidly raises oral pH to 10 (Thomas & MacLennan 1992).

Municipal drinking water in some geographical areas has been shown to be alkaline, with pH values even >9.5 (Kujala-Räty *et al*. 1998). Alkaline substances are also used in dental care. Bicarbonate toothpaste has pH in the range of 8–9.4. Calcium hydroxide is used as a therapeutic agent for deep cavities and pulp wounds to accelerate repair (Torneck *et al*. 1983). However, practically no studies on oral mucosal reactions to high alkalinity have been reported.

Prompted by the awareness of these alkaline conditions in the oral cavity and the suggested potential mitogenic properties of alkaline pH, the present study was designed to assess the effects of long-term exposure to alkaline drinking water (containing $Ca(OH)_2$ or NaOH) on the morphology of oral epithelium and other organs in rats. In addition, the expression of an extensive panel of proteins involved in keratinocyte differentiation, cell proliferation, cell adhesion, and cell stress was analysed using oral mucosal biopsy samples.

Materials and methods

Rats of the Long-Evans strain (both sexes) were maintained for one year under controlled environmental conditions (relative humidity 45–55%), with a 12-h light and 12-h dark cycle at 20–22 °C. The rats were housed in solid-polycarbonate bottom metal wire cages with alderwood bedding in groups of three. The study and control animals as well as females and males were separated. The animals were provided with standard laboratory animal feed (SDS, RM3, Essex, UK). Water was always available ad libitum. The water given to the rats in the study groups was modified to meet the criteria of the study design.

Forty-eight rats were divided into four study groups and one control group as Table 1 shows. $Ca(OH)_2$ (Merck, Darmstadt, Germany) or NaOH (Merck, Darmstadt, Germany) was used to raise water pH, measured using a pH meter (Radiometer PH M80, Copenhagen, Denmark). Water intake was monitored intermittently in all groups, showing no differences between groups. Similarly, food intake was equal in the study and control rats.

In groups 1, 2 and 3, the experiment was started when the rats were 12 weeks old, and in groups 4 and 5 at the age of 6 weeks. The duration of the experiment in all groups was one year (52 weeks), corresponding to an exposure of 30 years in humans. After completion of the study, the rats were killed using carbon dioxide, and necropsy samples were taken from the oral buccal mucosa, palate, tongue, oesophagus, stomach, intestines, liver, and kidney. In groups 4 and 5, additional samples were obtained from the salivary glands (parotis, submandibularis, sublingualis), masseter muscle, thyroid, hypothalamus, and ovary or testes. The samples

were fixed in 10% neutral formalin, embedded in paraffin, cut into 5-μm sections, and stained with haematoxylin and eosin for routine light microscopic evaluation. All biopsy samples were examined by a board-certified pathologist (KS), blinded for all other data.

Immunohistochemistry

For immunohistochemistry (IHC), formalin-fixed, paraffin-embedded samples were sectioned to 5-μm thickness and mounted on organosilan-coated slides. The sections were stained using the avidin-biotin complex (ABC) technique and an automatic appliance (DAKO Chem-Mate TM 500, BioTek Solutions, USA), according to the manufacturer's instructions. The antibodies used were pankeratin, cytokeratin (CK) 19, CK5, CK4, proliferating cell nuclear antigen (PCNA), intercellular adhesion molecule-1 (ICAM-1), CD44, CD68 and S-100, as well as heat shock protein (HSP) 60, HSP 70, and HSP 90. Table 2 lists the origin and dilution of the antibodies, as well as the pretreatment methods used for antigen retrieval. Appropriate positive controls were processed simultaneously with the study slides.

Results

Gross appearance and weight

All animals survived the experiment and remained in good condition until the end of the experiment. However, female rats, especially in Group 2 but also in Group 4 and 5, had dull-appearing and thin fur in patches at the end of the experiment. Some light-brownish discoloration of the oral mucosa was seen in Group 2 rats.

All animals were weighed at the completion of the experiment. The results are shown in Table 3. The mean weight of the rats in all experimental groups was lower (1–29%) than that of control animals. The lowest bodyweight was observed in rats receiving $Ca(OH)_2$ (Figure 1) and in those with exposure starting at low age (6 weeks) Thus, the weights of the rats in experimental groups differed statistically significantly from the controls (Kruskall-Wallis test) in the following groups: female rats of Group 2, female rats of Group 4, female and male rats of Group 5.

Microscopic evaluation

Oral mucosa

On microscopic examination, the epithelium of the oral buccal mucosa, palate, and tongue of the rats from the

Table 2. Dilution of antibodies and pretreatment of samples for immunohistochemical analyses

Antibody	Clone	Dilution	Pretreatment	Manufacturer	Reactivity
Pankeratin	MNF116	1 : 50	Microwave + prot-K	Dako, Glostrup, Denmark	Cytokeratin 5,6,8,17 and probably 19; stratified squamous epithelium and simple glandular epithelium
CK19	RCK108	1 : 100	Microwave + prot-K	Dako, Glostrup, Denmark	Simple and nonkeratinizing stratified epithelia
CK5	C-50	1 : 50	Microwave + prot-K	Monosan, Uden, Netherlands	Basal cells; simple and stratified epithelia
CK4	6B10	1 : 10	Microwave + prot-K	Monosan, Uden Netherlands	Non-cornifying epithelia, ductal epithelium
PCNA	PC10	1 : 100	Microwave	Pharmingen, San Diego, CA, USA	Proliferating cells
ICAM-1*	1A29	1 : 100	Microwave + prot-K	Pharmingen, San Diego, CA, USA	Activated mononuclear leucocytes, fibroblasts, epithelial and vascular endothelial cells
CD44*	OX-49	1 : 50	Microwave + prot-K	Pharmingen, San Diego, CA, USA	Activated lymphocytes, role in inflammation, repair and cell migration
CD68	KP1	1 : 150	Microwave + prot-K	Dako, Glostrup, Denmark	Macrophages/monocytes
S-100	polyclonal	1 : 1500	Microwave + prot-K	Dako, Glostrup, Denmark	Melanocytes, Schwann cells, lipocytes, dendritic reticulum cells, myoepithelial cells
HSP 60	LK2	1 : 25	No pretreatment	Sigma, St.Louis, MI, USA	Stress proteins induced by environmental perturbations such as elevated temperatures
HSP 70	BRM22	1 : 250	No pretreatment	Sigma, St.Louis, MI, USA	
HSP 90	AC-16	1 : 200	No pretreatment	Sigma, St.Louis, MI, USA	

* mouse antirat monoclonal antibody

Table 3. Comparison of weight data in control and study rats

Group:	Gender	Number of rats	Mean weight (g)	Weight range (g)	SD (+/–)	Mean weight compared with controls (g)	*P*-value (Kruskall-Wallis)	*P*-value (with Bonferroni correction)
1. Control	Female	6	233	212–240	10.56			
	Male	6	420	382–468	32.37			
2. Ca(OH)2, pH 11.2	Female	6	221	210–227	6.06	− 12	0.0370	0.148
	Male	6	373	322–432	48.22	− 47	0.1269	0.5076
3. NaOH, pH 11.2	Female	6	229	216–255	85.98	− 4	0.3358	1.3432
	Male	6	416	347–471	41.78	− 4	1.0000	4.0000
4. Ca(OH)2, pH 12	Female	3	206	187–207	5.68	− 27	0.0196	0.0784
	Male	3	372	357–385	14.19	− 48	0.0518	0.2072
5. NaOH, pH 12	Female	3	199	187–207	10.79	− 34	0.0196	0.0784
	Male	3	325	315–341	14.22	− 95	0.0201	0.0804

study groups was similar in morphology and structure to that of controls. No defined mucosal atrophy or ulcerations were observed in any of the rats. One of the rats in Group 2 showed moderate hyperkeratinization of the epithelium. Inflammatory cell infiltrates primarily consisting of lymphocytes in the lamina propria of the buccal mucosa were observed in all groups except Group 5. In the tongue samples, lymphocytic infiltrations were most prevalent in the control samples. In a male rat from Group 4, multinucleated giant cells were present in the buccal mucosa. Mast cells were most abundant in Group 3 rats both in samples from the buccal mucosa and tongue. A male rat in Group 3 and a female rat in Group 4 showed calcifications in the tongue.

Extraoral tissues and organs

In samples obtained from extraoral tissues, no marked changes were seen after alkaline exposure (data not shown). In the salivary glands, lymphocyte infiltration was present in the rats of the control Group 1 and in Group 3 and 4. In Group 2, no inflammatory changes were seen, but 3/12 (25%) of the salivary gland samples showed atrophic features. In the small intestine, one rat of Group 3 showed calcifications. In calcium hydroxide treated rats, one (8%) of Group 2 rats showed atrophy of the intestinal villi, and a granuloma was detected in a female rat of Group 4. Calcifications were seen in the kidney in 5 (42%) rats of Group 3, and also in 67% of the control rats.

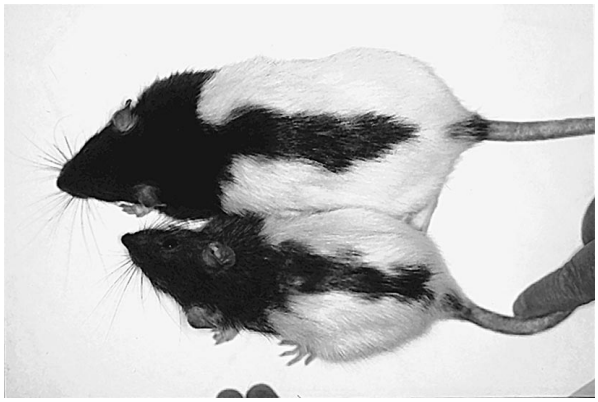

Figure 1. The largest female control rat (above) compared to a female rat from group 2.

Immunohistochemical analysis of buccal and palatal mucosa

Table 4 summarizes the results of the IHC analysis. Pankeratin, which detects keratins 5, 6, 8, 17 and 19, was strongly expressed throughout the whole epithelium in all samples in every study group. Epithelia showed intense staining of all supra-basal layers with

Table 4. Positive immunohistochemical staining in oral mucosal samples from study groups and controls

Antibody	Group 1 (Controls)	Group 2	Group 3	Group 4	Group 5
Pankeratin	12/12	12/12	12/12	6/6	6/6
CK 19	0/12	0/12	0/12	0/6	0/6
CK 5	12/12	12/12	12/12	6/6	6/6
CK 4	12/12	12/12	12/12	6/6	6/6
PCNA	12/12	12/12	12/12	6/6	6/6
HSP 60	6/12	5/12	8/12	3/6	5/6
HSP 70	4/12	12/12	7/12	5/6	4/6
HSP 90	12/12	12/12	11/12	6/6	6/6
S-100	12/12	12/12	12/12	6/6	6/6
ICAM-1	0/12	6/12	0/12	0/6	0/6
CD44	12/12	10/12	12/12	3/6	3/6
CD68	0/12	0/12	0/12	0/6	0/6

both CK4 and CK5, whereas all oral mucosal samples were totally negative for CK19. No differences in the intensity or quantity of expression were seen among the groups. PCNA with staining confined to the basal layer of the epithelium, showed neither differences in the quantity nor intensity of expression between the groups.

Weak expression HSP60 was seen in the nucleus of the basal cells, most frequently in Group 5 (83%) and in Group 3 (67%). Nuclear HSP 70 expression was mainly localized to the basal cells, present in 12/12 (100%) samples of rats in Group 2 and in 5/6 (83%) in Group 4 (treated with calcium hydroxide). In rats given sodium hydroxide in the water, HSP 70 was seen in 7/12 (58%) of the rats in Group 3 and in 4/6 (67%) of Group 5. In controls, HSP 70 expression was only seen in 4/12 (33%) samples.

Of the cell adhesion molecules, ICAM-1 did not stain in 6/12 samples from rats given $Ca(OH)_2$ pH 11.2 (Group 2). The antibody to CD44 used in this study recognizes both standard CD44 and splice variants (CD44v). All samples from the controls and Group 3 rats stained positive for CD44, whereas half of the samples in Group 4 and Group 5 remained CD44-negative.

Discussion

This study investigated the systemic (organ) and local (oral mucosal) effects of long-term administration of alkaline drinking water in rats. Parenchymal organs and oral mucosal samples were examined for morphologic changes, and the latter also for the expression of proteins regulating keratinocyte differentiation, cell proliferation, intracellular adhesion, and cell stress.

At the end of the one-year experiment, rats exposed to alkaline drinking water had significantly lower body weights than the controls (Table 3). In addition, the dull and patchy fur seen in some of the test animals suggests a systemic toxic or metabolic response to alkaline drinking water. However, the underlying mechanisms responsible for such a response are not readily recognizable on the basis of the present findings. Thus, we failed to observe any systematic gross or morphologic changes in any of the internal parenchymal organs or tissues examined on light microscopy. This does not, however, exclude the possibility of other factors, e.g. systemic atrophy of these organs, which can be reliably recognized only by quantitative measurements, which were not done in this study. The body weights were lowest in rats starting the ingestion of alkaline water at the age of 6 weeks. In humans, metabolic acidosis in children may lead to growth failure as reported in children with tubular acidosis (Chan 1983). Thus, it can be speculated that the increase in alkalinity in young rats could affect electrolyte or aldosterone metabolism responsible for the observed growth retardation. Of particular interest in this respect is the role of $Ca(OH)_2$, the effects of which on body weight were particularly pronounced in female rats (Table 3).

There are, however, some previous studies suggesting that an alkaline environment may have various effects at the cellular level. The dramatic effects seen after accidental ingestion of alkaline substances such as lye are well established (Howell 1986). Similarly, degeneration, haemorrhage and erosion of the gastric mucosa, and submucosal oedema have been reported in rats 15–60 min after administration of alkaline detergent formulations (Sauers *et al*. 1994). Treatment with 0,3N NaOH has been used experimentally to induce gastric ulcers in rats (Cheon *et al*. 1999). However, no histological change in the gastrointestinal tract was observed in this study, suggesting an adaptive response, which may include mucus stimulation, bicarbonate secretion, increased mucosal resistance, as well as gastric motility and increased gastric blood flow (Cheon *et al*. 1999).

Previous studies have shown that an alkaline extracellular environment may influence the growth properties of cells. Short periods (2–10min) of alkaline exposure (pH from 8.5 to 10) by the addition of NaOH in the cell culture medium have been shown to have a mitogenic effect on mouse embryo fibroblasts without any signs of toxic effects (Zetterberg & Engström 1981; Zetterberg *et al*. 1982). Similarly, the water extract of lime (Jeng *et al*. 1994) as well as hydroxy apatite and calcium carbonate crystals (Cheung & McCarty 1985) have been shown to increase the proliferation of cultured fibroblasts. A study showing transformation of canine kidney cells to dedifferentiated tumour cells only after exposure to a moderately alkaline environment (pH 7.8) for two weeks (Oberleithner *et al*. 1991) indicates profound effects of alkalinity on cellular growth and proliferation. No systemic organ changes, such as fibroblast proliferation (scarring), were observed in any of the parenchymal organs in the present study.

Much of the focus of the present study was on the assessment of local effects of alkaline drinking water on oral mucosa, by analysing the morphology and expression of cell regulatory proteins as previous study data implicate alkaline treatment as a cause of various local effects on the oral mucosa. Thus, in rats or hamsters, painting the oral mucosa with calcium hydroxide paste

regularly over a long period may lead to cell atypia but not malignancy (Malhotra 1993; Dunham *et al*. 1966; Sirsat & Kandarkar 1967; Gothoskar *et al*. 1975; Jin *et al*. 1996). Users of betel quid with alkaline slaked lime regularly develop deposition of fibrous tissue in the submucosal layers of the oral mucosa (Cox & Walker 1996). In the present series, however, no such fibrous changes or epithelial cell atypia were observed in alkaline-treated rats. No effect on the cell proliferation rate was seen, as evidenced by identical PCNA expressions in the study groups and control rats. This finding is in agreement with the totally negative staining of keratin CK19, the supra-basal expression of which is associated with epithelial proliferation (Depondt *et al*. 1999). Our findings are thus in line with the results of an *in vitro* study that failed to show any induction of cell proliferation by alkaline treatment as measured by counting the cells and thymidine incorporation (Burroni & Ceccarini 1984). In another study, snuff and alkaline snuff (pH 9.3) modified by sodium carbonate failed to induce any changes attributable to alkalinity in rat oral mucosa (Hirsch & Johansson 1983). One explanation for the absence of marked changes in rat oral epithelium in this study may be that the influence of alkaline water is short-lived because of its rapid clearance from the oral mucosa. Thus, the effect of alkalinity with calcium hydroxide paste application may well be more pronounced, owing to the irritating effect of the powder and its retention in the mucosa. The pH of rat saliva is also high, between 8 and 9 (Hirsch 1983), which could also have influenced the results of this study. Furthermore, the oral epithelium of the rat is generally keratinized and may therefore be resistant to the effects of exogenous irritative agents.

Of some interest was the effect of alkaline exposure on the expression of the adhesion molecules CD44 and ICAM-1. The expression of the hyaluronan receptor CD44 was slightly weaker in intensity in the experimental groups and completely absent in half of the rats receiving water with the highest pH. Several studies have shown that a down-regulation of this cell surface adhesion molecule and its different splice variants signify malignant transformation of oral epithelia (Soukka *et al*. 1997; Kuo *et al*. 1998). Reduced epithelial staining of CD44 has been associated with cell proliferation and mitotic activity (Hirvikoski *et al*. 1999), whereas the up-regulation of some isoforms such as CD44v9 may be involved in tissue differentiation (Stoll *et al*. 1999). No such connection between cell proliferation and loss of CD44 staining could be shown in the present study.

Another adhesion molecule, ICAM-1 was expressed in half of the CaOH pH 11.2 -treated rats, without any oral ulcerations. ICAM-1 expression is associated with inflammatory reactions and cell injury, induced by inflammatory mediators such as IL-1 and the pro-inflammatory cytokine, TNF-α (Patarroyo & Makgoba 1989). Furthermore, cellular stress may induce the expression of this adhesion molecule in keratinocytes. This may be a more plausible explanation in this context, because HSP70 expression was also up-regulated in the specimens, but no inflammatory reaction was present. Indeed, HSPs are biomarkers of exposure and cellular injury, and they are up-regulated in response to elevated temperature and chemical or physiological stress (Wu 1995; Sens *et al*. 1997). Furthermore, increased HSP 70 expression has also been found in benign, dysplastic, and malignant oral lesions (Sugerman *et al*. 1998). HSPs were analysed in the present study to evaluate the possible protective response to cellular stress due to this alkaline treatment. The up-regulation of HSP70 was seen especially in $Ca(OH)_2$-treated rats. This up-regulation may be an indicator of increased mucosal resistance to injury, as shown by the HSP72-dependence of resistance of the gastric mucosa to damage due to long-term aspirin administration in rats (Jin *et al*. 1996).

To conclude, our results indicate that the oral mucosa of Long-Evans rats remains relatively unaffected by high alkalinity without any specific morphologic alterations. On the other hand, long-term exposure to alkaline drinking water seems to have profound systemic effects manifested as significant growth retardation, as a result of mechanisms that require further studies.

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